PATENT
Docket No. 236472000720

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5-20-97
Date

Jennifer L. Taylor

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In the application of:

Michael C. Kiefer et al.

Serial No.: 08/320,157

Filing Date: October 7, 1994

For: NOVEL APOPTOSIS-MODULATING PROTEINS, DNA ENCODING THE PROTEINS AND METHODS OF USE THEREOF

Examiner: Ray F. Ebert

Group Art Unit: 1806

**DECLARATION UNDER 37 C.F.R § 1.132**

Assistant Commissioner for Patents
Washington, D.C. 20231

Dear Sir:

I, Michael C. Kiefer, declare as follows:

1. I am an inventor of the above-referenced patent application, and as such am familiar with the claimed subject matter. I have read the Office Action dated December 3, 1996 and understand that the Examiner would like to see evidence that CDN proteins of the claimed invention 1) accumulate intracellularly 2) are in a form that is available and bindable by antibodies made against recombinantly expressed CDN protein and 3) are present in sufficient quantity for detectable binding to antibodies. The following experiments were performed at my direction and show that CDN proteins are present in sufficient quantities and are recognized by

antibodies raised to recombinant CDN protein. A copy of my Curriculum Vitae is attached as Exhibit 1. As shown in my curriculum vitae, I am eminently qualified to supervise these experiments and to interpret the results.

2. Recognition of CDNs by anti-CDN antibody; Experimental protocol.

In these studies, the molecular basis of the effect of interferon-γ (IFN-γ) to increase the sensitivity of some cells to tumor necrosis factor-α- and anti-Fas antibody-mediated cell death was analyzed. A human colon adenocarcinoma cell line, HT-29, was treated with IFN-γ and the expression of various genes and proteins involved in apoptosis was measured. In the experiments described herein, the protein designated Bak is identical to the protein referred to as CDN in the instant invention.

In an attempt to identify genes that may be involved in the increased apoptotic sensitivity of HT-29 cells, the protein levels of various apoptosis modulators were analyzed before and after IFN-γ treatment. Whole cell extracts for Bak, Bax, Bcl-2 and Bcl-x analysis were prepared by lysing 1 x $10^7$ HT-29 cells in 200 µl of 1X phosphate-buffered saline (PBS) containing 1% NP-40, 0.5% Na-deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 1 mM phenyl methyl sulfonyl fluoride (PMSF), 10 µg/ml leupeptin and 1 µg/ml aprotinin for 10 minutes at 4°C. Lysates were centrifuged for 10 minutes at 1000 x g and supernatants were saved. For the protein determinations, lysates from HT-29 cells were quantitated by the Bradford assay. The samples (25-100 µg protein/lane) were resolved by 15% SDS-polyacrylamide gel electrophoresis (PAGE), transferred to nitrocellulose and incubated for 1 hr in blocking buffer (PBS containing 5% milk powder and 0.05% Tween-20). For Bak analysis, the blot was incubated for 1 hr with rabbit antisera directed against Bak in blocking buffer followed by a 1 hr incubation with anti-rabbit IgG-peroxidase conjugate (1:2000) and then developed using the ECL method (Amersham). The anti-Bak antisera was generated in rabbits using purified recombinant Bak produced in yeast and used at 1:5000 dilution. The anti Bcl-x, Bax and Bcl-2 antibodies were used as recommended by supplier (Santa Cruz Biotechnology, Inc.). The results obtained are depicted in Figure 1.

3. Results.

Northern blot analysis of bcl-2 family members had revealed that bak mRNA levels were induced after 16 hr of IFN-γ treatment, whereas the levels of bax, bcl-x, and bcl-2 mRNA were

unchanged. Bak protein levels also increased in HT-29 cells following IFN-γ treatment as shown by Western blot analysis (Fig. 1). At time zero, Bak protein levels were low but detectable.

The increase in Bak protein reflects the corresponding increase in Bak mRNA levels. Both Bax and Bcl-x protein levels remain unchanged following IFN-γ treatment which is consistent with their constant mRNA levels.

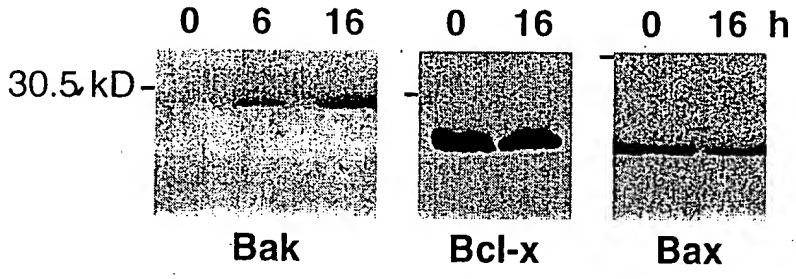


Fig. 1 Western blot analysis of Bak, Bcl-x and Bax protein levels. HT-29 cells were treated with 200 U/ml IFN-γ. At zero, 6 or 16 hours after treatment, cells were lysed and 25-100 μg total protein was loaded per lane onto a 15% SDS polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose filters. After blocking the filters, antibodies to either Bak, Bcl-x or Bax were added and allowed to bind. After washing the filter, peroxidase-labelled secondary antibodies were added and, after washing, detected by enhanced chemiluminescence, using a kit (Amersham).

The experiments described herein demonstrate that CDN proteins are intracellular proteins and can be detected in cell lysates. The experiments further demonstrate that antibodies generated using recombinant CDN protein produced in yeast can recognize CDN proteins in cell lysates, but not Bcl-x or Bax. These antibodies therefore recognize CDNs, but not other members of the bcl family tested.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to by true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

4-23-97 Michael C. Kiefer

Date Michael C. Kiefer

# MICHAEL C. KIEFER, Ph.D.

## PERSONAL


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| Home address: | 401 Wright Court, Clayton, CA 94517 |
| Home phone: | (510) 672-3576 |
| Business address: | LXR Biotechnology Inc., 1401 Marina Way South, Richmond, CA 94804 |
| Business phone: | (510) 412-9100 |


## EDUCATION

**1983 Ph.D. Biochemistry**
University California, Davis

**1976 Concurrent Undergraduate**
University California, Berkeley

**1974 B.S. Zoology, Graduated with Honors**
University California, Davis

## CAREER EXPERIENCE

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| **3/93 to 3/97** | **LXR Biotechnology Inc.**<br>Richmond, California |
| 2/96 to 3/97 | Vice President, Molecular Biology |
| 3/95 to 1/96 | Director, Molecular Biology |
| 3/93 to 2/95 | Senior Scientist |

Responsible for preclinical molecular biologial research and development, including strategic decisions, budgeting and research planning in the following areas:

— Gene Discovery: Identification, cloning and characterization of novel apoptosis-related genes and validation as important therapeutic targets.

— Signal Transduction: Identification and characterization of apoptotic pathways involving novel genes and drug leads discovered at LXR.

— Assay Development: Design and development of assays suitable for high-throughput screening of small molecule libraries with the goal of isolating compounds that modulate the apoptotic activity of genes, gene products and other lead drugs discovered at LXR.

Responsible for molecular biology presentations: 1) to pharmaceutical companies and other potential investors; and 2) at international scientific meetings.

Manage and direct a research group of 12 scientists, including 5 Ph.D.s. Research has resulted in 1 issued patent and 5 patents pending.

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| **7/87 to 2/93** | **Chiron Corporation**<br>Emeryville, California |
| 2/91 to 2/93 | Senior Scientist |
| 8/87 to 1/91 | Research Scientist |

Primary responsibility was discovery research in the areas of bone growth and differentiation. Utilized novel and standard cloning techniques to isolate cDNAs encoding new growth factor receptors, bone growth modulators, proteases and malarial antigens. The genes were expressed in yeast, baculovirus or mammalian based expression systems and the functions of the purified recombinant proteins were analyzed in appropriate *in vitro* cell based assays or *in vivo* animal models.

Research resulted in three issued patents and two patents pending.

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| **1/86 to 7/87** | **E.I. Du Pont de Nemours & Co., Medical Products & Immunology**<br>Glenolden, Pennsylvania |
| | Visiting Scientist |

Responsible for cloning and expressing genes involved in T and B cell differentiation and function. Primary emphasis was in utilizing cDNA expression vectors and expression systems which, in conjunction with differential cDNA screening, allow identification of the following: 1) Genes that are involved in T cell activation in response to interleukin 2 (IL-2); 2) pre-B cell specific surface antigens; and 3) lymphokines and lymphokine receptors. Other work included characterization and sequencing of two pre-B cell related cDNAs and gene transfer of the IL 2 receptor constructs into fibroblasts for structure/function analysis.

**10/83 to 12/85** **Basel Institute for Immunology**
Basel, Switzerland

Staff Scientist

Studied the molecular biology of T cell lymphocytes. Primary emphasis was the isolation and characterization of T cell specific genes including the T cell receptor. Approaches included subtractive cDNA cloning of T cell specific mRNAs. One T cell specific cDNA was further characterized by restriction enzyme analysis, Northern and Southern blot hybridization, DNA sequencing and chromosome mapping. The cDNA was expressed in bacteria as a fusion protein, purified and injected into rabbits to yield antisera that detected the protein in T cells.

**10/81 to 9/83** **U.S. Department of Agriculture, Plant Virology Laboratory, Plant Protection Institute**
Beltsville, Maryland

Postdoctoral Researcher

Investigated the mechanisms of viroid replication and pathogenesis. Approaches used were the following: 1) Comparisons of the primary and secondary structure (derived by computer analysis) of viroids that display a wide range of symptoms in tomato. The sequence of two viroids were determined by direct enzymatic RNA sequencing and chemical sequencing of cDNA clones. 2) Analysis of viroid replication and pathogenesis in tomato, using as inocula, potato spindle tuber viroid and tomato apical stunt viroid cDNA clones, and chimeric and mutant derivatives.

**10/76 to 9/81** **University of California, Davis, Dept. of Biochemistry**
Davis, California
Major Professor - Dr. George Bruening

Doctoral Researcher

Investigated the molecular basis of plant resistance to virus infection using a resistant variety of *Vigna unguiculata* (cowpea) protoplasts and cowpea mosaic virus as a model system. Techniques used included liposome mediated delivery of viral RNA into protoplasts, ELISA assay, Northern blotting, *in vivo* radiolabelling of protein and nucleic acids, gel electrophoresis and fluorescent antibody labelling of virus infected protoplasts.

**12/75 to 9/76** **University of California, Berkeley, Dept. of Biochemistry**
Berkeley, California

Research Professor - Dr. Michael Chamberlain

Predoctoral Researcher

Investigated early transcriptional termination in bacteriophages T3 and T7. Techniques used included *in vivo* radiolabelling of virus specific proteins, gel electrophoresis and phage and bacterial selection and growth.

**10/74 to 9/75** **University of Freiburg, Institut für Biologie III**
Freiburg, West Germany
Research Professor - Dr. Gerd Hobom

Predoctoral Researcher

Duties included purification of restriction endonucleases, restriction mapping of bacteriophages, growth and purification of defective bacteriophage lambda and agarose gel electrophoresis.

**3/73 to 9/74** **University of California, Davis, Dept. of Biological Chemistry**
Davis, California
Research Professor - Dr. Robert Traut

Undergraduate Researcher

Investigated the specificity of ATP and GTP dependent protein kinases with respect to their ability to phosphorylate ribosomal proteins of *Escherichia coli.* Techniques used included ribosome purification by zonal centrifugation, two dimensional gel electrophoresis and enzyme assays.

## TEACHING EXPERIENCE

**9/77 to 7/80** **University of California, Davis, Dept. of Biochemistry and Biophysics**
Davis, California

Teaching Assistant

Led discussion sections in undergraduate biochemistry laboratory and lecture courses.

## PUBLICATIONS


1. Issinger, O.G., Kiefer, M.C., and Traut, R.R. (1975). Specificity of ATP-dependent and GTP-dependent protein kinases with respect to ribosomal proteins of *Escherichia coli.* Eur. J. Biochem. *59*, 137-143.

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**INVITED SPEAKER**

American Chemical Society Meeting, San Francisco, April 1992. "Characterization of recombinant insulin-like growth factor binding proteins -4, -5, and -6 produced intracellularly in yeast."

Ninth International Congress on Endocrinology, Workshop on IGF binding proteins, Opio, France, August 1992. "Characterization of recombinant insulin-like growth factor binding proteins -4, -5, and -6 produced intracellularly in yeast."

American Society for Biochemistry and Molecular Biology Fall Symposium, Genetic and Biochemical Approaches for Studying Cell Death, Granlibakken, Lake Tahoe, California, October 1994. "Cdn-l,-2, and-3, a family of Bcl-2-related genes that modulate apoptosis."

Fourth Abano Terme Meeting on Rehabilitation, First International Conference on Apoptosis in Skeletal and Cardiac Muscles (ASCM96), Abano Terme (Padova), Italy, June 1996. "Interferon-γ sensitizes cells to apoptotic stimuli and increases apoptosis-related gene expression."

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